

BMJ Open Polymorphisms of genes related to vitamin D metabolism and transportation and its relationship with the risk of osteoporosis: protocol for a multicentre prospective cohort study in China

Jing Wang ^{1,2,3}, Bing Shu,^{1,2,4} Chen-Guang Li,^{1,2} Xing-Wen Xie,⁵ De Liang,⁶ Bo-Lai Chen,⁷ Xin-Chao Lin,⁸ Xu Wei,⁹ Liang Wang,¹⁰ Xiang-Yang Leng,¹¹ Ying-Jie Zhou,¹² Pei-Zhan Chen,¹³ Yu-Ren Tao,^{1,2} Yong Zhou,¹⁴ Yan Zhang,^{1,2,4} Xue-Jun Cui,^{1,2} Sheng Lu,^{1,2,4} Hui Wang,¹⁵ Qi Shi,^{1,2} Yong-Jun Wang^{1,2,4,16}

To cite: Wang J, Shu B, Li C-G, *et al.* Polymorphisms of genes related to vitamin D metabolism and transportation and its relationship with the risk of osteoporosis: protocol for a multicentre prospective cohort study in China. *BMJ Open* 2019;**9**:e028084. doi:10.1136/bmjopen-2018-028084

► Prepublication history for this paper is available online. To view these files, please visit the journal online (<http://dx.doi.org/10.1136/bmjopen-2018-028084>).

Received 22 November 2018
Revised 18 October 2019
Accepted 22 October 2019



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For numbered affiliations see end of article.

Correspondence to

Dr Yong-Jun Wang;
yjwang8888@126.com

ABSTRACT

Introduction Osteoporotic fracture is one of the most common causes of disability and a major contributor to medical care costs in many regions of the world. The polymorphisms of genes related to vitamin D metabolism and transportation are associated with variation in bone mineral density and the risk of osteoporosis.

Methods and analysis The China Community-based Cohort of Osteoporosis study is an observational, longitudinal, multicentre, prospective cohort study for middle-aged and older permanent residents of China, which has been ongoing in six cities since 2016. Female residents aged 45–80 years old and male residents aged 50–80 years old are identified through permanent resident lists. All the enrolled participants will complete questionnaires on their personal characteristics and histories. The bone mineral density of their lumbar vertebrae and left hip will be measured and serum bone metabolism parameters assessed. Polymorphisms of genes related to vitamin D metabolism and transportation will be detected, and their relationship with the risk of osteoporosis, and osteoporotic fracture, will be analysed. About 18 000 residents will be involved in the study.

Ethics and dissemination The study was approved by Institutional Ethics Board of Longhua Hospital affiliated to Shanghai University of Traditional Chinese Medicine (2016LCSY065). Results will be published in peer-reviewed journals. The results of this study are expected to improve the understanding of the association between polymorphisms of genes related to vitamin D metabolism and transportation and the risk of osteoporosis and osteoporotic fracture among middle-aged and older residents of China.

Trial registration number NCT02958020

BACKGROUND

Osteoporosis is a growing problem worldwide because of the huge medical expense of

Strengths and limitations of this study

- This is the first nationwide community-based longitudinal study in China on the polymorphisms of genes related to vitamin D metabolism and transportation and their relationship with the risk of osteoporosis.
- A movable dual energy X-ray absorptiometry instrument will be used to facilitate the measurement of bone mineral density and improve the response rate.
- The questionnaires do not include detailed dietary information, because of the time limits of a large-scale on-site study.
- Fractures without significant clinical symptoms may be misdiagnosed and lead to the detection signal bias.
- This is a multiregion cohort study, and the genetic diversity among the populations may be a confusion variable.

fractures, especially low-energy injuries (slips, trips and falls). Osteoporotic fracture is one of the most common causes of disability and a major contributor to medical care costs in many regions of the world. A recent study found that low-energy injuries caused 66% of fractures in older men and 83% of fractures in older women, and suggested that osteoporotic fractures are now a major problem in China.¹ Better awareness of osteoporotic lesions may improve prevention and treatment of osteoporosis.

Vitamin D, an essential nutrient for humans, has multiple functions in bone metabolism.^{2–4} It is widely accepted that vitamin D deficiency is associated with skeletal disorders, including

osteopaenia, osteoporosis and the risk of fractures.^{4 5} Vitamin D is synthesised from 7-dehydrocholesterol by skin cells following exposure to sunlight, and can be converted into its biologically active form by 25-hydroxylase (CYP2R1) in the liver and 25-hydroxyvitamin D-1 α -hydroxylase (CYP27B1) in the kidney.⁶ About 85%–90% of circulating 25-hydroxyvitamin D is bound to vitamin D binding protein (DBP, encoded by the GC gene), which transports it between tissues.⁷ Vitamin D can bind to the widely expressed nuclear vitamin D receptor (VDR) and regulates about 3%–5% of human gene expression.⁸

Research has shown that polymorphisms of genes related to vitamin D metabolism and transportation, including the genes for VDR, DBP and CYP2R1, are associated with variation in bone mineral density (BMD) and the risk of osteoporosis. A study in Thailand⁹ found that VDR gene polymorphism was associated with osteoporosis in postmenopausal Thai women. Another showed that the allelic variants of VDR gene polymorphism were significantly correlated with reduction in BMD, which resulted in increasing risk of osteoporosis in postmenopausal North Indian women.¹⁰ Two polymorphisms in the GC gene may be linked to higher BMD in black Americans than their white peers, even though they also had less 25-hydroxyvitamin D.¹¹ Polymorphisms in CYP2R1-rs10766197 are also associated with vitamin D deficiency in Uyghur and Kzazk populations living in China.¹²

China is a vast low/middle-income country with regional and national differences in genetic background, geographical position and cultural environment. However, there has been no large sample cohort study on the polymorphisms of genes related to vitamin D metabolism and transportation, and their association with the risk of osteoporosis. The China Community-based Cohort of Osteoporosis (CCCO) is an observational, longitudinal, multicentre, prospective cohort study among middle-aged

and older permanent residents in six cities in China. The study is designed to improve understanding of the associations between polymorphisms of genes related to vitamin D metabolism and transportation and the risk of osteoporosis in the study population.

METHODS AND ANALYSIS

Subcentre selection

CCCO was initiated in 2016, and is being conducted in nine centres across six cities. The cities are in the east, west, middle, south, north and north-east of China. The hospital characteristics for the selected sites are shown in [table 1](#). These sites were selected based on the abilities of the local investigators to administer the survey consistently, the existence of suitably qualified doctors to diagnose osteoporosis and relatively fixed communities in those regions. Each community has a group of committee members who are responsible for routine administration service, which facilitates the organisation of the study. There is a fixed area dedicated to the CCCO study in each community health centre, with research staff from the corresponding grade III hospital.

Participant recruitment and sample size calculation

Female residents aged 45–80 years old and male residents aged 50–80 years old are identified through permanent resident lists. Lactating or pregnant women and residents with mental health problems, acute infectious diseases and severe physical diseases who cannot cooperate with the investigations are excluded. Enrolled residents are notified by the community residents' committee and signed the relevant consent forms. Residents who respond and complete the first survey will be resurveyed annually for the next 5 years, and then every 5 years later. Non-respondents will be followed up with a series of postcard reminders and telephone interviews.

Table 1 Characteristics of hospitals participating in CCCO

City, province (Locations in China)	Site	Survey district scope
Shanghai (East)	Longhua Hospital Affiliated to Shanghai University of Traditional Chinese Medicine	Longhua, Lujiazui and Hangtuo Districts
Lanzhou, Gansu (West)	Gansu Provincial Hospital of Traditional Chinese Medicine	Wuquan and Fuerlu Districts
Luoyang, Henan (Middle)	Luoyang Zhenggu Hospital of Henan Province	Junan Community
Guangzhou, Guangdong (South)	The First Hospital Affiliated to Guangzhou University of Traditional Chinese Medicine	Xicun District
Guangzhou, Guangdong (South)	Guangdong Provincial Hospital of Traditional Chinese Medicine	Guangta District
Beijing (North)	Wangjing Hospital of China Academy of Chinese Medical Sciences	Xiangheyuan District
Beijing (North)	The 309th Hospital of Chinese People's Liberation Army	Hangcaiyuan District
Beijing (North)	Dongzhimen Hospital, Beijing University of Chinese Medicine	Dongzhimen District
Changchun, Jilin (Northeast)	Hospital Affiliated to Changchun University of Traditional Chinese Medicine	Dongzhan, Nanhu and Qinghe Districts

CCCO, China Community-based Cohort of Osteoporosis.

Table 2 Items included in baseline and follow-up questionnaires

Item	Questionnaire
Participant characteristics and risk factors	Demographic characteristics, Chinese ethnic nationality, marital status, level of education, family income, type of career, pregnancy and parity history, age at menarche and menopause, frequency and amounts of daily smoking and alcoholic drinking, dietary habits, daily hours of sleep and sun exposure.
Fracture history	Time, fracture location (hip, spine, wrist and other non-vertebral sites, that is, clavicle, upper arm, shoulder, forearm, rib, pelvis, ankle, femur, tibia and fibula), causes and treatment of fracture; number of falls in the past years.
Medications for osteoporosis (currently taking or ever taken)	Bone medications; calcium; vitamin D; oestrogen or hormone replacement; cortisone or prednisone.
Comorbidities (ever diagnosed) and related medications	<ol style="list-style-type: none"> 1. Cardiovascular or cerebrovascular conditions: cerebral infarction, cerebral haemorrhage, coronary disease, hyperglycaemia and hypertension.; 2. Endocrine conditions: diabetes, hyperthyreosis, hypothyroidism, hyperparathyroidism and gout. 3. Degenerative bone disease: prolapse of lumbar intervertebral disc, lumbar spinal stenosis and knee osteoarthritis. 4. Urologic diseases: chronic kidney diseases and kidney transplantation. 5. Digestive system disease: hepatitis, cirrhosis, hepatic adipose infiltration, liver transplantation, alcoholic liver disease, gastritis and gastric ulcer. 6. Other medical conditions: rheumatoid arthritis, systemic lupus erythematosus and cancer.
Physical activity	IPAQ-SF.
Quality of life	EQ-5D.
Osteoporosis risk	One minute test of the International Osteoporosis Foundation.

These examinations focus on possible causes and consequences of osteoporosis.

EQ-5D, EuroQol-5 dimension; IPAQ-SF, short form of the International Physical Activity Questionnaire.

We estimated the sample size based on the incidence of osteoporosis morbidity in the Japanese population-based osteoporosis study. The estimated prevalence of osteoporosis is 36.1%¹³ in women aged 20–79 years old, based on total hip BMD, and 4%¹⁴ in men aged 20–79 years old. A sample size estimation method was used with random sampling and proposed $\alpha=0.05$, and $\beta=0.10$. The minimum sample size was estimated as 9219, so we plan to recruit 18000 participants, almost twice the required minimum sample size. This will require approximately 3000 participants in each region.

Questionnaires

All the participants will complete paper-based questionnaires (see [table 2](#)) and undergo an extensive set of examinations through face-to-face interviews. The questionnaires include survey date, participant characteristics and related risk factors, including comprise demographic characteristics, Chinese ethnic nationality, marital status, education level, family incomes, occupation, pregnancy and parity history, menstrual history, frequency and amount of daily smoking and alcohol drinking, frequency of urination and defecation, dietary habits, hours per day of sleep and sun exposure, bone fracture history and medical history. To evaluate dietary habits, participants will be asked to recall the frequency and amount of major food groups eaten in a week, including vegetable and animal oil, rice, cooked wheat-based food, coarse cereals, salted food, meat, poultry, eggs, seafood, freshwater fish and shrimp, animal viscera, bean products, vegetables,

fruit, milk and milk products, tea, coffee and carbonated drinks. Medical history, including diagnosis, medication use and disease status for comorbidities, will be obtained from participants' medical records.

Physical activity will be evaluated using the short form of the International Physical Activity Questionnaire (IPAQ).¹⁵ Osteoporosis risk will be evaluated by the 1 min test of the International Osteoporosis Foundation¹⁶ and life quality will be evaluated with EuroQol-5 dimension.¹⁷

Physical examinations

Height and body weight will be measured for body mass index (BMI) calculation. The waist circumference and hip circumference will be recorded, and the ratios calculated. The body fat contents will be measured with the dual energy X-ray absorptiometry densitometer (DEXA, Hologic Discovery CI, Bedford, Massachusetts, USA). The muscle strengths of both hands will be measured with a hand dynamometer. The five-times-sit-to-stand test will be used to evaluate the participants' coordination. Each of these measures will be assessed twice and the average score used in analyses.

Assessment of BMD and osteoporosis diagnosis

The BMD of the participants' left hip, and each lumbar vertebra, and the average BMD values (g/cm^3) of lumbar vertebra (L1-4) will be measured using a DEXA instrument. The same instruments will be used in all the centres. BMD values will be expressed as T-scores (number of SD above/below the mean for the patient's age or for healthy



30 years old adults). The Chinese Society of Osteoporosis and Bone Mineral Research states that a clinical diagnosis of osteoporosis can be made in postmenopausal women and men aged 50 and over who sustain a low-trauma fracture (such as hip, vertebra or radius fracture), or when the spine and hip BMD are less than or equal to 2.5 SD below the young normal mean (T-score ≤ -2.5) at any bone site, even a single vertebra. For premenopausal women and men under the age of 50, a diagnosis of osteoporosis is established by spine and hip BMD of less than or equal to 2.0 SD below people of the same race, sex and age (Z-score ≤ -2.0) at any bone site, even a single vertebra.

Blood sample collection

Each participant will provide a total of 7 mL of venous blood after an overnight fast (10 hours). The blood samples will be collected in both ethylene diamine tetraacetic acid (EDTA) anticoagulation and non-EDTA tubes to collect plasma and serum samples. The serum samples will be collected within 2 hours of the blood collection at room temperature. After being collected in EDTA-containing tubes, the blood will be gently mixed and centrifuged at 3000 rpm for 15 min at room temperature to separate the plasma. The plasma and serum will be stored in aliquots at -80°C until use. A range of haematological and biochemical tests will be conducted on fresh samples at the central laboratory of King Med Diagnostics, a large testing company with 37 medical laboratories across China.

Tests will be carried out to measure the levels of osteocalcin, β -C-terminal telopeptide of type I collagen, N-terminal propeptide of type I collagen, alkaline phosphatase,

total serum 25(OH)D (25(OH)D₃ and 25(OH)D₂), total serum calcium and phosphorus, parathormone, fibroblast growth factor 23 and serum concentration of pyridoxal 5'-phosphate (vitamin B₆). The detection methods and instruments are shown in table 3.

Genomic DNA will be extracted from the lymphoid cells using the standard phenol-chloroform method. The purified DNA will be stored at -20°C until use.

Genomic polymorphism analysis

Genomic DNA of blood cells will be extracted and preserved in a refrigerator at -80°C for genetic polymorphism analysis. The single nucleotide polymorphisms of related genes, including GC gene and genes of VDR and vitamin D metabolic enzymes (table 4), will be genotyped using PCR-restriction fragment length polymorphism.

Follow-up

A long-term follow-up will be performed every year until 2021. After that, follow-up will be carried out every 5 years depending on the research funding. The follow-up questionnaires are identical for all follow-up periods. The first 5-year follow-up questionnaires will include changes in marital status, family income, occupation, menstrual status, medical diagnosis and treatment, daily smoking and alcohol intakes, food habits and history of falls. Physical activities will be evaluated using the long form of the IPAQ.¹⁸

BMD measurement and physical examinations will be performed annually. Information about new fractures will be obtained from participants either by self-reports or from interviews during the follow-up visits. Participants

Table 3 Serological detection method

Index	Serological detection	Detecting instrument
OST	Electrochemiluminescence immunoassay	Automatic biochemical analyzer (cobas 8000 e602, Roche)
β -CTX	Electrochemiluminescence immunoassay	Automatic biochemical analyzer (cobas 8000 e602, Roche)
PINP	Electrochemiluminescence immunoassay	Automatic biochemical analyzer (cobas 8000 e602, Roche)
PTH	Electrochemiluminescence immunoassay	Automatic biochemical analyzer (cobas 8000 e602, Roche)
ALP	Continuous monitoring technique	Automatic biochemical analyzer (modular P800, Roche)
FGF23	ELISA	Micro plate reader (MK3, Thermo, Waltham, Massachusetts, USA)
Vitamin B ₆	High efficiency liquid chromatography	Ultra-performance liquid chromatograph (Agilent 1290 Infinity, Santa Clara, California, USA)
25(OH)D	High performance liquid chromatography–tandem mass spectrometry	Liquid chromatography tandem mass spectrometry (API4000, AB SCIEX, Framingham, Massachusetts, USA)
Ca	O-cresolphthalein-complexone method and phosphomolybdate ultraviolet colorimetry	Automatic biochemical analyser (modular P800, Roche)
P	O-cresolphthalein-complexone method and phosphomolybdate ultraviolet colorimetry	Automatic biochemical analyzer (modular P800, Roche)
DBP	ELISA	Micro plate reader (MK3, Thermo, Waltham, Massachusetts, USA)

ALP, alkaline phosphatase; Ca, serum calcium; β -CTX, β -C-terminal telopeptide of type I collagen; DBP, vitamin D binding protein; FGF23, fibroblast growth factor 23; 25(OH)D, 25-hydroxyvitamin D₃ and D₂; OST, osteocalcin; P, serum phosphorus; PINP, propeptide of type I collagen; PTH, parathormone.

Table 4 Single nucleotide polymorphisms of genes

Protein	Genetic locus of SNPs
GC	rs4588, rs7041, rs222020, rs2282679
VDR	rs1544410, rs2239181, rs21077301, rs2239179, rs2189480, rs3819545, rs2239186, rs2254210, rs2238136, rs4760648, rs11168287, rs4328262, rs4334089, rs3890733, rs110783219, rs7299460
Vitamin D metabolic enzymes (CYP2R1, CYP27B1)	rs10741657, rs2060793, rs12794714, rs10877012

SNPs, single-nucleotide polymorphisms; VDR, vitamin D receptor.

with fractures will be asked to describe the causes of fracture (slips, trips or falls, traffic accidents, crushing injury, sharp or blunt trauma and others) and provide their medical records (reports of imaging examinations and treatment). We will define fractures (at any bone site) caused by low-energy injuries (slips, trips and falls) as osteoporotic fractures.

Outcomes

The primary outcome of this study is the changes in BMD values for the participants. The differences in BMD values between the yearly follow-up points and the baseline will be calculated as changes in BMD values. These will be used to evaluate the association between polymorphisms of genes related to vitamin D and BMD changes. The secondary outcome is the occurrence of osteoporotic fracture, which will be used to analyse the relationship between gene polymorphisms and the risk of osteoporotic fracture.

Inquirer quality assurance

All research assistants, interviewers and physical examiners will be trained centrally by professional epidemiologists and experienced staff at the central coordinating centre over a 2-week period with repeat training per 2 years. Training will be conducted onsite, and within the laboratories of each of the participating investigators under the supervision of experienced staff, until the required standard of testing and competency has been achieved. Observer variations will be assessed at the start and midpoint of the study and the anthropometric equipment calibrated annually at the start of the survey.

Data management

Completed questionnaires will be sent to the central coordinating centre, to be scanned and saved electronically. The data entry software is designed to detect outliers, inconsistencies, and omissions and to document their resolution. Scanned data will be entered into a database stored in a secured password-protected computer. As a quality control measure, each study site will maintain an administrative database to track survey questionnaires. Two meetings will be held annually with each study

coordinator to review the survey administration and ensure uniformity of the process.

Statistical analysis

As first step, the characteristics of the study participants (such as age, BMI, etc) will be shown as the medians and the IQR or number and proportion. The distributions of the parameters will be shown by mean and SD. The proportions of participants with osteoporosis, osteopaenia or normal bone mass will also be listed. For the diagnosis of osteoporosis or osteopaenia, categorised BMD values (T or Z values) will be used, and the continuous BMD values (g/cm^3) will be used in the rest analysis.

Evaluation of the primary outcome

For the baseline data, the normal distribution of parameters (such as biochemical parameters) will be compared between groups using analysis of variance. Pairwise comparison of multiple groups will be performed with least significant difference test when homogeneity of variances are satisfied, and Dunnett's test for heterogeneity of variance. Comparison of categorical variables (such as genotype, smoking, alcohol drinking and comorbidities) will use the χ^2 test. Post hoc testing for the difference between pairs of genotype groups will use Tukey's method to test whether the genotype distribution is consistent with the Hardy-Weinberg equilibrium. The correlation between the variants will be determined using Pearson's coefficient of correlation. The relationships between BMD changes and other continuous variables (such as biochemical parameters) will be examined using univariate linear models.

Binary regression analyses or multivariate linear regression analyses will be used to identify risk factors associated with unfavourable outcomes (such as gender, age, BMI, quartile interval or specific scope of biochemical parameters, genotype, smoking, alcohol drinking, comorbidities and physical activities). The correlation between the polymorphisms and BMD changes will be determined using Spearman's correlation coefficient.

Evaluation of the secondary outcome

A longitudinal assessment of related risks for the incidence of osteoporosis or osteoporotic fracture among participants with different SNP genotypes will be analysed using a survival analysis with Kaplan-Meier curves. Cox multivariate regression models will be used to compare the probability of osteoporosis or osteoporotic fracture in the follow-up cohorts, adjusting for the necessary covariates (such as gender, age, BMI, biochemical parameters, genotype, smoking, alcohol drinking, comorbidities and physical activities). The relative risk (HRs) will be calculated with a 95% CI. The level for statistical significance will be set at $\alpha=0.05$ (two tailed).

In all the above analyses, stratification analysis on certain characteristics including gender and age (in 10-year intervals) will be performed.



Patient and public involvement

Patients included in the study provided self characteristics and related risk factors of osteoporosis. No patients were involved in setting the research question, nor were they involved in developing plans for recruitment, design or implementation of the study. No patients were asked to advise on interpretation or writing up of results. The results will be informed through community health promotion organisations, which were established by the government.

DISCUSSION

Osteoporosis can result in both morbidity and mortality, and is also related to huge medical expense around the world. Early intervention and preventive strategies are very important to decrease the occurrence of osteoporosis and osteoporotic fracture in older people. A cohort study of osteoporosis involving 60 393 postmenopausal women aged 55 years and over in 10 countries and three continents suggested that there might be regional differences in the proportion of hip fractures.¹⁹ There are also differences in both incidence and prevalence of fractures across geographical regions and countries.^{20–23} In China, therefore, it is important to identify regional differences in the prevalence of osteoporosis and osteoporotic fracture to develop specific prevention approaches.

The polymorphisms of genes related to vitamin D metabolism and transportation are associated with BMD and the risk of osteoporosis.^{9–12} In China, there are regional and national differences in polymorphisms of these genes, which may lead to different levels of risk of osteoporosis and osteoporotic fracture.^{24–25} This study aims to compare the genotype distributions of vitamin D related genes in middle-aged and older participants in different regions of China. We will also evaluate the effect of these gene polymorphisms on risk of osteoporosis and osteoporotic fracture and assess the interactions of gene polymorphism and other factors during the development of osteoporosis, including dietary habits, physical activity and environmental factors.

This study has several strengths, including its large sample size and representativeness. CCCO will involve participants in six cities across the north, south, north-east, west, east and middle of China so can be considered to represent most Chinese communities. Large community-based prospective cohort studies are essential for the unbiased assessment of the relevance and interaction between environmental and genetic factors. DEXA will be used to measure the participants' BMD, which is installed on a medium bus. This improves the convenience of community screening. However, his study also has several limitations. First, it will be conducted in a real-world setting, which may lead to variable information quality, and its clinical value will be lower than a randomised controlled study. Second, recall bias may arise from some of the retrospective questions, such as disease history and changes in

medications. Third, we are proposing to recruit large populations from different regions of China, however, the genetic diversity among the populations could be a confusion variable.

Author affiliations

- ¹Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China
- ²Key Laboratory of Theory and Therapy of Muscles and Bones, Ministry of Education, Shanghai, China
- ³Shanghai Geriatric Institute of Chinese Medicine, Shanghai, China
- ⁴Spine Institute, Shanghai University of Traditional Chinese Medicine, Shanghai, China
- ⁵Gansu Provincial Hospital of Traditional Chinese Medicine, Lanzhou, China
- ⁶The First Hospital Affiliated to Guangzhou University of Traditional Chinese Medicine, Guangzhou, China
- ⁷Guangdong Provincial Hospital of Traditional Chinese Medicine, Guangzhou, China
- ⁸Dongzhimen Hospital, Beijing University of Chinese Medicine, Beijing, China
- ⁹Wangjing Hospital of China Academy of Chinese Medical Sciences, Beijing, China
- ¹⁰The 309th Hospital of Chinese People's Liberation Army, Beijing, China
- ¹¹Hospital Affiliated to Changchun University of Traditional Chinese Medicine, Changchun, China
- ¹²Luoyang Zhenggu Hospital of Henan Province, Luoyang, China
- ¹³Clinical Research Center, Ruijin Hospital North, Shanghai Jiao Tong University School of Medicine, Shanghai, China
- ¹⁴Beijing Institute of Heart, Lung and Blood Vessel Diseases, Beijing Anzhen Hospital, Capital Medical University, Beijing, China
- ¹⁵School of Public Health, Shanghai Jiao Tong University School of Medicine, Shanghai, China
- ¹⁶University of Traditional Chinese Medicine, Shanghai, China

Acknowledgements We thank Melissa Leffler, MBA, from Liwen Bianji, Edanz Editing China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

Contributors Y-JW and QS conceived the review. JW, BS, YZ, X-JC, X-WX, DL, B-LC, X-CL, XW, LW, X-YL, Y-JZ, P-ZC, Y-RT, C-GL and SL performed clinical investigation. JW and YZ undertook the analyses. JW and BS wrote the initial draft. Y-JW and HW critically revised the manuscript.

Funding This study was funded by the National Key R&D Program of China (2018YFC1704300), National Nature Science Foundation of China (81730107 to Y-JW, 81673991 to BS and 81574001 to C-GL), Shanghai TCM Medical Center of Chronic Disease (2017ZZ01010 to Y-JW), National Clinical research base of TCM project (JDZX2015075 to C-GL, JDZX2015076 to XW, JDZX2015077 to X-YL, JDZX2015078 to DL, JDZX2015079 to X-CL, JDZX2015080 to X-WX, JDZX2015081 to B-LC, JDZX2015082 to Y-JZ), the Program for Innovative Research Team of Ministry of Education of China (IRT1270 to Y-JW), the Program for Innovative Research Team, Ministry of Science and Technology of the People's Republic of China (2015RA4002 to Y-JW), Three Years Action to Accelerate the Development of Traditional Chinese Medicine Plan (ZY(2018-2020)-CCCX-3003 to Y-JW) and Guanghua Excellent PI Team (2016-06 to Y-JW).

Competing interests None declared.

Patient consent for publication Not required.

Ethics approval The study was approved by the Institutional Review Board at Longhua Hospital affiliated to the Shanghai University of Traditional Chinese Medicine (2016LCSY065).

Provenance and peer review Not commissioned; externally peer reviewed.

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ORCID iD

Jing Wang <http://orcid.org/0000-0002-4611-2003>

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